Two genes encoding distinct cytosolic glutamine synthetases are closely linked in the pine genome[1]

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Abstract The major isoenzyme of glutamine synthetase found in leaves of angiosperms is the chloroplastic form. However, pine seedlings contain two cytosolic glutamine synthetases in green cotyledons: GS1a, the predominant isoform, and GS1b, a minor enzyme whose relative amount is increased following phosphinotricin treatment. We have cloned a GS1b cDNA, and comparison with the previously reported GS1a cDNA sequence indicated that they correspond to separate cytosolic GS genes encoding distinct protein products. Phylogenetic analysis showed that the newly reported sequence is closer to cytosolic angiosperm GS than to GS1a, suggesting therefore that GS1a could be a divergent gymnospermous GS1 gene. Gene mapping using a F2 family of maritime pine showed co-localization of both GS genes on group 2 of the genetic linkage map. This result supports the proposed origin of different members of the GS1 family by adjacent gene duplication. The implications for gymnosperm genome organization are discussed.

Key words: cDNA sequence; Conifer; Cytosolic glutamine synthetase; Gene family; Gene mapping; *Pinus*

1. Introduction

Nitrogen availability is usually a limiting factor for tree growth and development. Nitrate is the most common form of inorganic nitrogen to be utilized by the plant, except in acidic soils of boreal forests where little nitrification occurs and ammonium is predominant [1]. Conifers, unlike herbaceous plants, have a preference for ammonium over nitrate as nitrogen source [2,3]. Plant metabolic activity also releases ammonium in many different processes. Both assimilation from the soil and reassimilation into biomolecules are crucial processes for plant nitrogen economy which are catalyzed by glutamine synthetase (GS, EC 6.3.1.2).

There are two types of GS in angiosperms, which are found in different cellular compartments, GS1 in the cytosol and GS2 in the chloroplasts [4]. GS2 is encoded by a single nuclear gene in most species [5]. Expression of GS2 in leaves is restricted to photosynthetic mesophyll cells. GS1 is encoded by a small gene family of several members exhibiting different patterns of gene expression during development and in response to external stimuli [6]. More uncertain are the specific biological roles of individual GS1 gene products. Recent reports indicate that they are involved in primary assimilation of ammonium from the soil [7], reassimilation of ammonium released in the biosynthesis of lignin [8] and nitrogen mobilized during senescence [9], in response to pathogen attack [10], herbicide treatment [10,11] or water stress [12].

The members of the GS1 gene family in plants have been proposed to have evolved by duplication of an ancestral cytosolic GS gene and evolution of separate gene copies to fulfil different metabolic cell requirements [13]. GS2 genes are much more similar to cytosolic GS genes than to prokaryotic GS genes and therefore it is assumed that they originated by evolution of a duplicated GS1 copy rather than by transfer from the chloroplast [14].

We are interested in the characterization of genes involved in nitrogen assimilation in conifers. Seedlings of pine and other conifer species mainly express cytosolic GS in both photosynthetic and non-photosynthetic tissues [15]; however, functional expression of the GS2 gene has not yet been demonstrated in a reliable way. These inferences were assessed by the immunocytochemical detection of GS1 in mesophyll and phloem cells of pine seedlings [16], suggesting a key role for cytosolic GS in the early development of conifers. It remains to be determined whether the same or different gene products (GS1) are present in both mesophyll and phloem cell types in pine.

We have previously characterized a GS1 cDNA clone from Scots pine [17] and showed that it encodes the predominant GS polypeptide in green tissues [18]. This gene is actively expressed in pine cotyledons in a light-dependent fashion [19] suggesting a specific role in glutamine biosynthesis and ammonium assimilation associated with chloroplast activity. However, data derived from Southern blotting suggested that Scots pine GS is encoded by a gene family of at least two members [17]. We recently reported the existence of two GS isoproteins in Scots pine, GS1a and GS1b. The additionally reported GS isoform, GS1b, has been identified in cotyledons of phosphinotricin (PPT)-treated plants [20]. This new

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GS exhibited a different chromatographic behavior and subunit composition but a similar size compared to the previously characterized cytosolic enzyme, GS1a. In this paper, the isolation of a full-length cDNA clone encoding GS1b from PPT-treated pine seedlings is described. A comparison of the cDNA sequences of GS1b and GS1a indicates that they correspond to separate cytosolic GS genes encoding distinct protein products. The two genes are evolutionarily divergent but are closely located in linkage group two of the maritime pine genetic map.

2. Materials and methods

2.1. Plant material

Scots pine seeds (*Pinus sylvestris*) were obtained from Servicio de Material Genético, ICONA (Instituto de Conservación de la Naturaleza), Guadalajara (Spain). Seed germination and growth of seedlings have been previously described [21].

2.2. RNA extraction and Northern blot analysis

Total RNA was isolated using phenol/SDS [22]. For Northern analysis, total RNA (10 μg per lane) was separated on denaturing formaldehyde–agarose gels and blotted onto nylon filters which were prehybridized at 42°C in 50% formamide, 5×SSC, 5×Denhardt's solution (1×Denhardt's solution is 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin), 50 mM sodium phosphate pH 6.5 and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed under the same conditions, but in the presence of 10% dextran sulfate at 42°C.

2.3. RT-PCR reaction, cDNA library construction and screening for GS clones

PCR (polymerase chain reaction) amplification was carried out as described [23], using a GeneAmp 2400 thermocycler (Perkin-Elmer). Beforehand, a cDNA was synthesized using poly(A)$^+$ RNA extracted from cotyledons of Scots pine seedlings treated with PPT [20]. A solution containing the herbicide (25 mM in Tween-20 0.02% v/v) was sprayed over plants and samples were harvested after treatment.

Poly(A)$^+$ RNA was purified from total RNA isolated from 2.5 cm cotyledons of PPT-treated seedlings using two sequential rounds of affinity chromatography on oligo(dT) columns [24]. cDNA synthesis was achieved using the Stratagene synthesis kit. The cDNA library was cloned into λZAP vector and plated on the host strain XL1 Blue. Plaque screening hybridizations were performed in a solution containing 6×SSC, 5×Denhardt's solution, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA at 65°C. Filters were washed twice in 2×SSC, 0.1% SDS for 30 min at 65°C followed by 0.2×SSC, 0.1% SDS for 30 min at 72°.

2.4. DNA sequencing and structural analysis

Restriction fragments derived from cDNA clones corresponding to the GS1b gene were subcloned into pBluescript SK to serve as templates for double-stranded DNA sequencing using primers T7 and T3. Nucleotide sequences were determined using the dideoxy chain termination method [25] and Sequenase enzyme (United States Biochemical Corporation). Computer sequence analysis was carried out using the Genetics Computer Group (GCG) software package [26]. Sequence analysis comparison was made using the Fasta program [27].

2.5. Phylogenetic analysis

We have analyzed 10 plant GS amino acid sequences corresponding to the coding regions of cytosolic and chloroplast isozymes from angiosperms as well as the *Pinus* sequences GS1a and GS1b. The *Chlamydomonas* sequence was used as an outgroup, and a partial sequence of another alga (*Dunaliella*) was also included. These are the only published sequences of non-flowering plants available. To study the phylogenetic relationships of the plant sequences with the *Chlamydomonas* GS2 sequence, we constructed an alignment of these sequences, and a number of fungi and prokaryotic sequences, using the *Escherichia coli* sequence as an outgroup.

The sequences were obtained from SwissProt or translated from the nucleotide sequences, and aligned using the program PileUp [26]. The compared stretch of the alignments was that overlapping with the 357 residues of the *Pinus* GS1a sequence. The phylogenetic analysis was carried out under the maximum parsimony criterion through the program PAUP version 3.1.1 [28]. Phylogenetic trees were obtained with the heuristic search option. Starting trees were obtained by stepwise addition with one tree held at each step. Tree bisection–reconnection branch swapping was performed with the MULPARS option. The reliability of the clades obtained was tested through a bootstrap analysis performed with the heuristic search option, taxa added at random order and 100 replicates [29].

The sequences included in the alignments, the key used in Fig. 3 and the accession numbers were as follows: Angiosperm GS1: *Lactuca sativa* (Lact, P23712), *Medicago sativa* (Med, P04078), *Nicotiana plumbaginifolia* (Nic, P12424), *Oryza sativa* (Ory, P14656), *Phaseolus vulgaris* (Phas, P04771). Angiosperm GS2: *Arabidopsis thaliana* (Ara2, S69727), *Nicotiana tabacum* (Nic2, S39536), *Oryza sativa* (Ory2, P14655), *Pisum sativum* (Pis2, P08281), *Zea mays* (Zea2, P25462). Gymnosperm: *Pinus sylvestris* GS1a (Pina, X69822), *Pinus sylvestris* GS1b (Pinb, AJ005119). Algae: *Dunaliella salina* (Dun, P11600), *Chlamydomonas reinhardtii* (Chl and Chl2, Q42688 and Q42689). Fungi: *Agaricus bisporus* (Agar, O00088), *Colletotrichum gloeosporoides* (Coll, Q12163), *Saccharomyces cerevisiae* (Sacc, M65157). Prokaryotes: *Bacillus subtilis* (Bac, P12425), *Escherichia coli* (Eco, P06711), *Methanococcus maripaludis* (Meth, E1309187), *Pyrococcus furiosus* (Pyr, Q05907), *Rhizobium leguminosarum* (Rhiz, P09826), *Synechocystis* 6803 (SynA and SynN, X69199 and X76719).

2.6. Two-dimensional electrophoresis of proteins and Western blotting analysis

Total proteins were extracted from needles and vascular tissue of *Pinus pinaster* separated by 2D PAGE and revealed by silver staining [30]. Alternatively, 2D gels were electrotransferred to nitrocellulose filters and the GS spots immunodetected using specific antibodies [18]. In situ proteolytic digestion of GS spots, peptide purification and microsequencing analysis were performed following the method of Costa et al. [31].

3. Results and discussion

Two GS1 isoforms (GS1a and GS1b) are present in Scots pine cotyledons [20]. GS1a is predominant in the tissue [17,32] whereas GS1b is a minor activity whose relative amount is increased following PPT treatment of seedlings [20]. These findings strongly suggested enhanced expression of an addi-

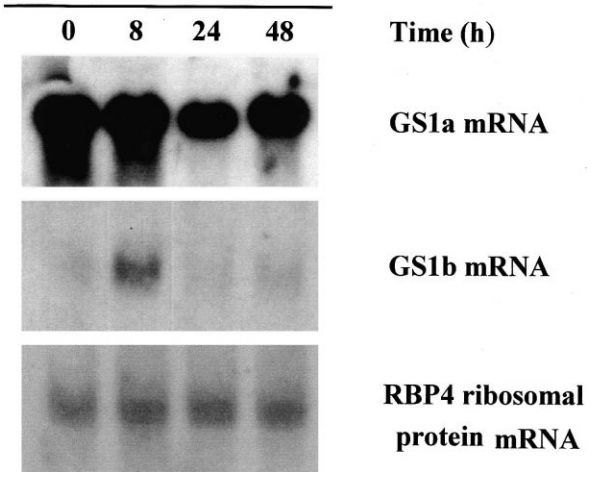

Fig. 1. Expression analysis of GS1a and GS1b genes in PPT-treated Scots pine seedlings. GS gene expression was studied in 2.5 cm cotyledon length plants treated with PPT after 8, 24 and 48 h. Time 0 h represents control untreated plants. Treatment conditions were as described in Section 2. 10 μg of total RNA was loaded per track and hybridized with GS1b and GS1a 3′-untranslated specific probes in each case. Time exposures to film were 24 h for GS1a and 8 h for GS1b. The same filter was reprobed with an RBP4 ribosomal protein DNA probe to compare the relative loading among samples.

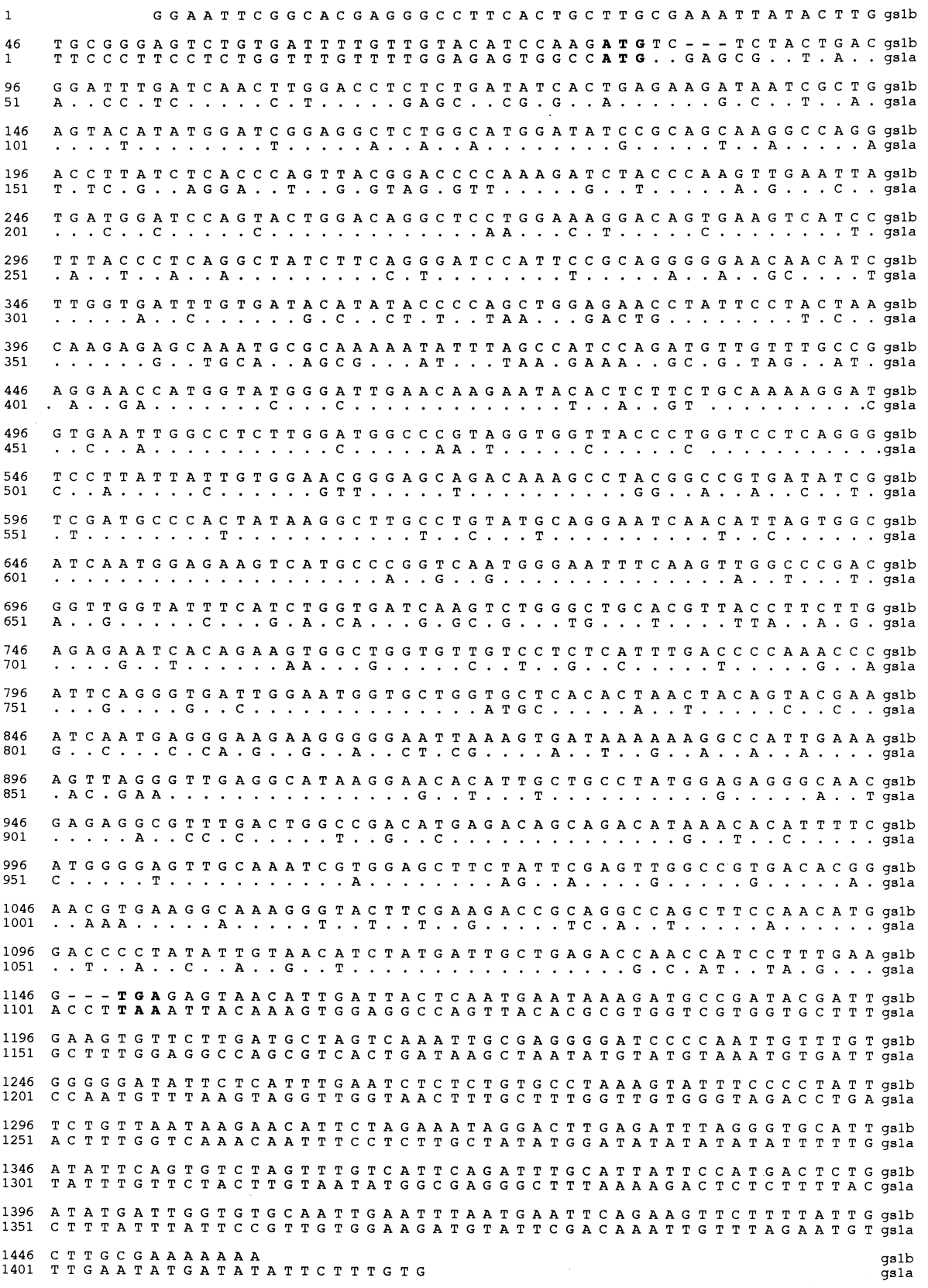

Fig. 2. Comparison of nucleotide sequences of Scots pine GS1b with the previously characterized GS1a clone [17]. The sequence of the GS1a cDNA coding region is only indicated when it differs from GS1b, except for initiation and termination codons which are marked in bold. Dashes indicate missing nucleotides in the alignment.

tional GS1 gene in the above conditions. To examine this possibility, RNA was isolated from cotyledons of PPT-treated plants and an apparent single band was amplified by RT-PCR using the following primers: 5′-GGTCGTCTCAGCAATCAT-3′ and 5′-GGCATCAATGGAGAAGTCATGCCAGGGCAGTGG-3′.

As conserved GS primers were used for DNA amplification, the PCR product presumably corresponded to a mixture of GS1 amplificates. The cloning and differential hybridization using the previously reported cDNA as a probe [17] allowed us to identify two kinds of positives that hybridized differently to the probe. Sequence analysis of both types of clones confirmed that the strongly hybridizing positives were 100% homologous in sequence to the pine GS cDNA described earlier [17], whereas the weakly hybridizing signals, quantitatively more abundant, corresponded to a GS sequence that was related but not identical to the previously cloned GS1a [17]. Using this PCR product as a probe, we obtained a full-length cDNA for GS1b (Section 2).

The strategy followed to detect this new type of cytosolic GS was a transient treatment of plants with PPT. To determine to what extent the GS1b message was affected by the herbicide treatment, we isolated total RNA from cotyledons of the treated plants 8, 24 and 48 h after treatment and GS message was assessed by Northern blotting analysis (Fig. 1). The relative abundance of GS1b mRNA was very low in untreated plants, as would be expected for an accessory isoenzyme in that tissue. Herbicide treatment induced GS1b gene expression in a transient way with a peak at 8 h and returned to initial levels after 24 h. No significant effect was observed in GS1a transcript abundance after treatment. These results are in agreement with previously reported data on the effects of PPT treatment on glutamine synthetase protein isoforms [20] indicating that enhancement of GS1b abundance is correlated with mRNA level. In tomato cotyledons and leaves PPT also triggered the appearance of an additional GS1 polypeptide [10,11].

The complete nucleotide sequence of GS1b (pGSP15) cDNA is 1451 bp in length with an open reading frame spanning 1068 nucleotides, a 5′-untranslated region of 77 nucleotides and a 3′-untranslated portion of 306 nucleotides including a poly(A) tail. A comparison of the nucleotide sequences of GS1b cDNA (pGSP15) and GS1a (pGSP114), the other GS1 cDNA from *P. sylvestris* previously reported [17], is presented in Fig. 2. The coding regions show a high degree of conservation, 73.4% identity, frequently using alternative codons for the same amino acid residues. However, no significant homology was found in the 5′- and 3′-untranslated regions indicating the cDNA sequences represent two different pine GS1 genes (Fig. 2).

Table 1
Characteristics of the predicted protein products of GS1b (pGSP15) and GS1a (pGSP114) Scots pine cDNAs

	GS1b	GS1a[a]
Amino acid sequence identity	100	81.5
Number of residues	355	357
Molecular mass (kDa)	39.2	39.5
Isoelectric point	6.0	6.6
Charge at pH 7.0	−4.0	−2.0
1 A_{280nm} (mg/ml)	0.6	0.6

[a]Data from Cantón et al. [17].

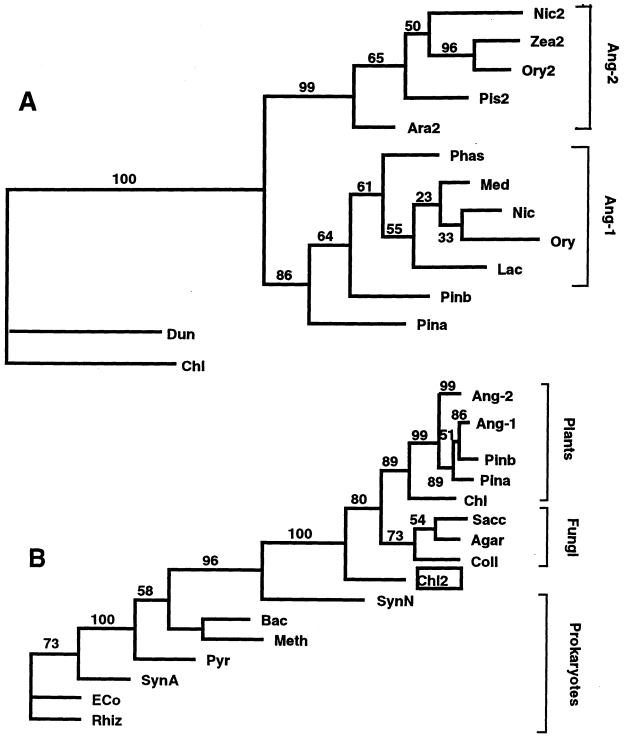

Fig. 3. Phylogenetic analysis of Scots pine GS1a (Pina) and GS1b (Pinb) sequences. The length of the branches is proportional to the number of changes along the branch. The numbers represent the percentage of bootstrap replicates supporting the clades. A: The maximum parsimony tree (536 steps, CI = 0.757) shows a distinct clustering of the sequences in two groups, consisting of the chloroplastidic (Ang-GS2) and cytosolic sequences (Ang-GS1). The *Pinus* sequences cluster with the angiosperm GS1 clade. B: The inclusion of prokaryote and fungus sequences shows that the *Chlamydomonas* GS2 sequence (boxed) is unrelated to the angiosperm GS2 gene, being the sister group of all the plant and fungus sequences. See Section 2 for key of sequence identification.

Some characteristics of the predicted protein products of GS1b (pGSP15) and GS1a (pGSP114) are shown in Table 1. The derived protein sequence for GS1b does not have a N-terminal pre-sequence, thereby identifying it as a cytosolic-like protein. This inference is supported by comparative protein sequence analysis (data not shown) indicating that the GS1b amino acid sequence is more similar to cytosolic GS (83–86% identity) than to chloroplastic GS enzymes (72–76%). However, the GS1b sequence is closer to angiosperm cytosolic GS than to pine GS1a (81.5%) (Table 1). The GS1b deduced protein sequence has 355 residues, two less than GS1a, resulting in a small decrease in molecular mass (39.5 to 39.2 kDa), insufficient to separate the polypeptides by SDS–PAGE. However, GS1b and GS1a with predicted isoelectric points of 6.0 and 6.6 respectively can be easily resolved by 2D PAGE [20]. These sequence-derived data are consistent with the observed GS profiles obtained by anionic exchange chromatography with cotyledon samples treated with PPT which indicated differences in the charge of both GS1 isoproteins [20]. The elution of GS1b at a higher ionic strength (0.5 M) than GS1a (0.15–0.18 M) is consistent with a higher content of acidic amino acid residues.

To examine explicitly the phylogenetic relationships between the genes, we performed a cladistic parsimony analysis of the combined nucleotide sequences of both gymnosperm

GS sequences with 23 previously published GS genes including angiosperms, algae, yeast and prokaryotes. When we only included the angiosperm sequences, and the compared stretch of the alignments overlapped with the 357 residues of the *Pinus* GS1a, we obtained a most parsimonious tree of 536 steps (Fig. 3A), with a consistency index of 0.757. The tree showed a definite separation of the plant GS sequences into two groups, cytosolic and plastidic. Bootstrap analysis showed the consistency of the GS2 and the (GS1a, GS1b, angiosperm GS1) clades, which appeared in 99% and 86% of the replications, respectively. Bootstrap proportions $\geq$70% correspond to a probability $\geq$95% that the respective clade is a historical lineage [33]. This evidence suggests that the gene duplication which created chloroplast GS from an ancestral cytosolic gene occurred long before the divergence gymnosperms/angiosperms. This would explain the grouping of the cytosolic *Pinus* sequences with the angiosperm GS1 sequences, the angiosperm GS2 sequences being a sister group. Although expression of the GS2 gene in conifers has not been detected [16,17,21], the above data are consistent with the recent finding of GS2 expression in *Ginkgo biloba* leaves [16].

With regard to the evolutionary relationships of cytosolic GS genes, *Pinus* GS1a and GS1b sequences clustered with the GS1 sequences, GS1a being the sister group of the (GS1b, angiosperm GS1) clade. This clade was present in 64% of the bootstrap replications, while the clade (GS1a, GS1b) only appeared in 21%, and the (GS1a, angiosperm GS1) in 6% of the replications. The results reveal the structural relationship between GS1b and cytosolic angiosperm GS rather than between the *Pinus* GS1 sequences. The higher consistency of the (GS1b, angiosperm GS1) clade suggests the possibility that angiosperms might have received an ancestral GS1 gene more closely related to the *Pinus* GS1b whereas GS1a is a unique gymnospermous GS1 gene. It is tempting to speculate on the possibility that the GS1a gene might be orthologous to the GS2 gene in angiosperms taking into consideration that tissue-specific expression has been suggested as a criterion to predict GS gene orthology [13]. In fact, recent data from our laboratory demonstrated that the nuclear gene for GS1a is expressed in green cotyledons in a light-dependent fashion [19] suggesting a specific role in glutamine biosynthesis associated with chloroplast activity.

In this context, the recent report of a putative GS2 gene in *C. reinhardtii* [34] prompted us to study the phylogenetic relationships with plant GS genes. Phylogenetic analysis of the *Chlamydomonas* GS2 sequence (Fig. 3B) places this gene midway between the prokaryotic and eukaryotic GS sequences, and phylogenetically unrelated to the plant chloroplastidic GS sequences. We think that the *Chlamydomonas* GS2 gene may result from duplication of an ancestral GS gene in primitive eukaryotes, although transfer from the plastid endosymbiont genome to the host cell genome cannot be excluded [35].

Thus, although more GS sequences from algae and lower plants are required to outline the evolution of GS isoforms, our findings strongly suggest that plant GS2 evolved from a duplicated GS1 gene around the time of land plant evolution, after separation of algae and vascular plants but before the separation of the angiosperm/gymnosperm lineages. This result would address the question of why a 'prokaryotic GS gene' has not been maintained in the nuclear genome during evolution of chlorophyta unlike other genes involved in nitrogen metabolism (i.e. Fd-GOGAT) or photosynthesis (RbcS, Lhcb2).

Recently, a genetic linkage map has been established in maritime pine (*P. pinaster*) using RAPD and protein markers [36–38]. Scots (*P. sylvestris*) and maritime (*P. pinaster*) pines are closely related species with presumably a high degree of conservation in gene structure and organization. This assumption has been demonstrated by PCR amplification of a 1.3 kb fragment using conserved primers for GS1 and genomic DNA from both pine species as template [39]. The DNA sequence of the obtained PCR products was homologous in 97% including

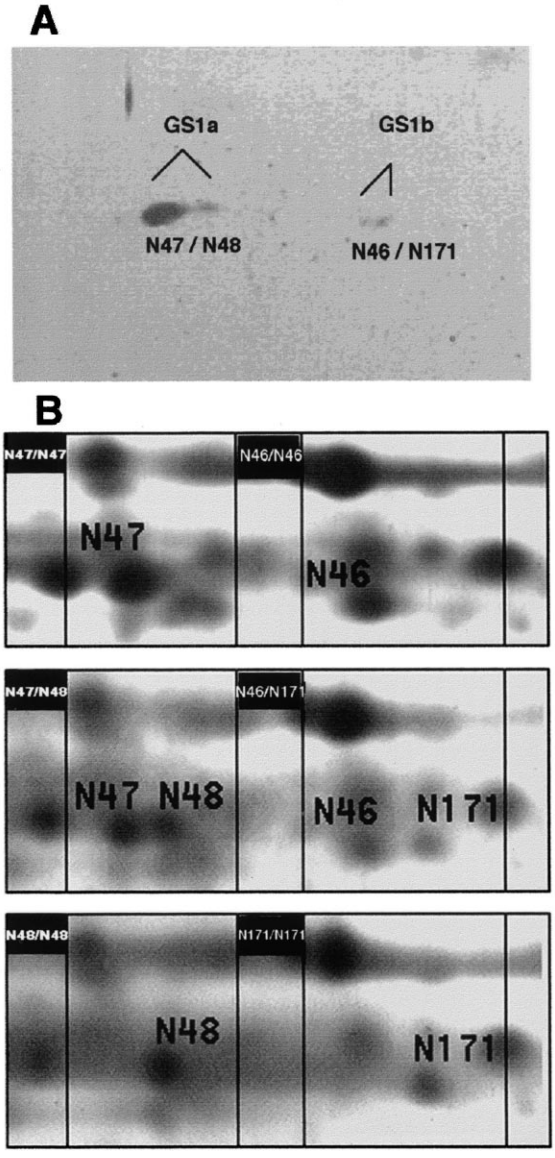

Fig. 4. Two-dimensional electrophoresis, protein microsequencing and segregation analysis of GS1a and GS1b expression products in maritime pine. A: Immunodetection of GS1a and GS1b protein spots using specific GS antibodies [18]. Note the presence of allelic products for both genes. B: Silver staining of total soluble protein showing a position shift variation for a pair of spots segregating in Mendelian fashion in a F2 (selfed) pedigree of maritime pine. Spots N47 and N48 are allelic products of GS1a (basic GS) and segregate in the 1/4:1/2:1/4 ratio, for genotypes (N47/N47), (N47/N48), and (N48/N48). Spots N46 and N171 are allelic products of GS1b (acidic GS) and segregate in the 1/4:1/2:1/4 ratio, for genotypes (N46/N46), (N46/N171), and (N171/N171).

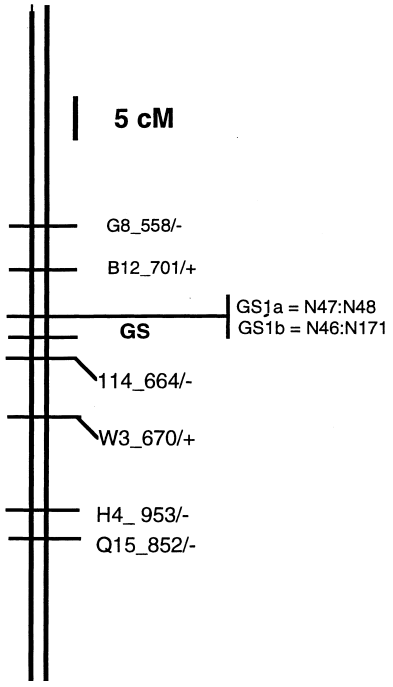

Fig. 5. Map location of the GS1 genes in linkage group two of the maritime pine genetic map [36]. GS1a, pair N47:N48 = S2205-87; GS1b, pair N46:N171

introns (data not shown) confirming the similarity of GS genes in both pine species.

We mapped GS1a and GS1b genes using a F2 family obtained by selfing one hybrid tree, a cross of the Landes and Corsican provenances of maritime pine. Protein products of the two GS1 genes have been detected in photosynthetic tissues (needles) of *P. pinaster* by 2D analysis and Western blotting (Fig. 4A) using GS antibodies raised against GS1 protein expressed in *E. coli* [18]. Two needle spots (named N47 and N48, see URL http://www.pierroton.inra.fr/genetics/2D) corresponding to two allelic products of GS1a were localized on linkage group 2 of the maritime pine genetic map [37,38]. A 15 amino acid microsequence of spot N47 (DVNWPLGWPVGGYPG) confirmed it was a glutamine synthetase protein. These spots were close together on a 2D gel, slightly differing in their p*I* (Fig. 4). Approximate molecular size and isoelectric point were 40–41 kDa and 6.5 respectively, consistent with expectations that the polypeptide corresponded to GS1a (Table 1). Two identical microsequences for glutamine synthetase protein (IIAEYWIGGSGMDI) were obtained from two immature xylem spots of maritime pine (X16 and X17, see URL http://pierroton.inra.fr/genetics/2D). Both spots were located close together on a 2D gel, slightly differing in their p*I*. This polypeptide was slightly smaller in size and had a lower p*I* value (5.7) than GS1a, suggesting it corresponded to GS1b (Table 1). Comigration analysis between xylem and needle proteins confirmed spot homology. In this photosynthetic tissue both GS spots (named N46 and N171) showed a positional polymorphism (Fig. 4B) allowing this protein to be placed on the maritime pine genetic map. Again this protein was localized to linkage group 2.

The more striking observation was that GS1a and GS1b exactly cosegregate, i.e. no recombinant could be found when 68 F2 individuals were analyzed on 2D gels. In addition, both GS were found to be located with the GS gene already mapped using the PCR-based approach [39].

The above data indicate that GS1a and GS1b genes are closely linked on linkage group two of the maritime pine genetic map (Fig. 5). This result agrees well with the proposed origin of different members of the GS1 family by a gene duplication phenomenon [14] suggesting the presence of both cytosolic GS genes as a tandem repeat in the pine genome. In fact, there is some experimental evidence showing that genes in some plants are not uniformly distributed in the genome, but localized in clusters separated by interspersed blocks of repetitive DNA sequences [40].

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